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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

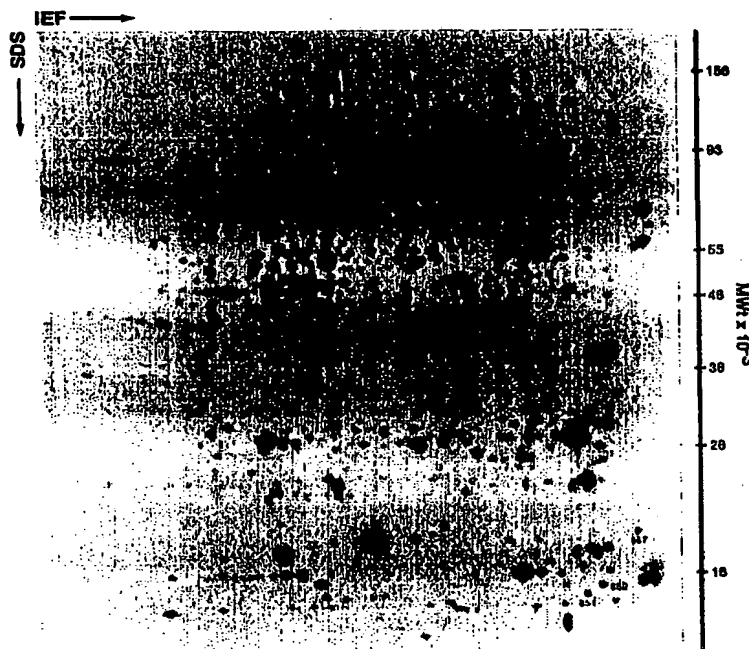
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(54) Title: HYPERTENSION MARKERS

## (57) Abstract

The present invention relates to a method of detecting for an individual mammal a high likelihood of having hypertension or a genetic predisposition for having hypertension, the method comprising determining the presence in a biological sample from the individual mammal of at least one marker protein which is indicative of a high likelihood of having hypertension or a genetic predisposition for having hypertension, wherein the marker protein is selected from the group consisting of the proteins shown in Table 1. In a further aspect the invention relates to novel proteins, nucleic acid fragments and use of said proteins or nucleic acid fragments for detection of whether an individual mammal has a high likelihood of having hypertension or a genetic predisposition for having hypertension as well as test kits for this purpose.

## HYPERTENSION MARKERS



● Down regulated from normotensive to hypertensive.  
● Up regulated from normotensive to hypertensive.  
Numbers are internal computer reference numbers.

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## HYPERTENSION MARKERS

## SUMMARY OF THE INVENTION

Apart from being a very serious complication in connection with, e.g., diabetes and various renal diseases, hypertension is in itself a very widespread disease which, in addition to having a lowering effect on the life quality, also directly leads to death of the patient. Hypertension may in fact be the cause of many different cardiac/vascular diseases (such as cardiac arrest, cerebral haemorrhage, and thrombi). It is unfortunately a disease which is currently increasing, and in spite of enormous resources being spent, research in this field has not reached (1) an understanding of the background for hypertension; (2) a clear definition of actual risk groups; and (3) an actual effective treatment. For a review, see Krieger and Dzau, 1991.

On this background, the present group of inventors started a project with the aim of finding the genetic background for hypertension, since it is obvious that, apart from influences from the surroundings, there is a clear genetic background for this disease.

There are no immediate useful models for studying hypertension; there are, however, several animal models (mainly based on rats) which may be used. Thus, normotensive WKY rats (i.e. rats having a normal blood pressure) were compared with hypertensive SHR rats (i.e. "Spontaneous Hypertensive Rats"). The way in which hypertension arises in SHR rats is very analogous to the conditions seen in human beings.

The part of the vascular and venous system which regulates the blood pressure consists of the small resistance vessels and thus, these very vessels were used in the present studies - other tissues and possibly organs (e.g. the kidneys) could also have been used. Before killing the rats in order to examine the resistance vessels, about 25 various physiologi-

cal parameters were measured in the animals (e.g. blood pressure, body temperature,  $\text{Ca}^{2+}/\text{Na}^{+}/\text{Mg}^{2+}$  concentrations) in order to ensure that the rats complied with the requirements in question. After the killing, proteome analysis studies  
5 were carried out on each of the isolated vessels from the rats, again to ensure that they were all optimum vessels.

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In order to make sure that the findings were really due to a genetic effect, it was not the parental rats (WKY and SHR) which were used for the analyses. Instead, a so-called "gene-  
10 tic backcrossing" was made (see Fig. 1): the WKY and SHR rats were crossed, yielding an F1 generation. These F1 rats were then crossed with each other in order to obtain an F2 generation, and the rats of this generation were used for the studies. As expected, this F2 generation showed a larger  
15 spreading of the blood pressures of the rats (see also Fig. 2) than the parental generation; some were normotensive (about one fourth) and some were very hypertensive (about one fourth).

The working hypothesis was: If there were differences in the  
20 expression of various genes (i.e. proteins expressed in various amounts in the small resistance vessels) when comparing the normotensive and the hypertensive F2 rats, and these differences were due to a genetic factor, then it should be possible to find the same differences between the  
25 WKY rats and the SHR rats. Of course, the argument could have been turned around and the point of departure taken in the parental generation; however, this is not the best method since these rats will differ from each other in a number of other ways than just the blood pressure (e.g. colour of the  
30 hair, colour of the eyes, fur type, etc.). Of course, all these phenotypic differences are not expressed in the resistance vessels, but they may very well disturb the actual changes which are due to differences in blood pressure. These other phenotypic differences are, for a large part, "crossed  
35 out" by using the F2 generation as the point of departure and then grouping the rats according to their blood pressures.

By labelling the small resistance vessels with [<sup>35</sup>S]-methionine for 20 hours, it was possible, in a reproducible manner, to follow and analyze no less than about 3500 proteins from each rat (32 F2 rats were used) by means of two-dimensional gel electrophoresis (see Fig. 3). All of the 2D gel images were scanned into an image processing computer, edited, matched and re-edited to eliminate mis-matches. The data (e.g. percentage integrated optical density for each spot) was exported into a spreadsheet and the blood pressures of the individual rats were statistically compared with the expression of each protein. A comparison of the normotensive and the hypertensive rats showed 59 proteins which were expressed significantly differently between the two blood pressure groups.

The same analysis was then carried out by comparing WKY rats with SHR rats; this gave 96 significant differences.

When considering the 10 most significant differences in the F2 generation, it is very interesting to see that four of these are also present as very significant differences in the comparison between the WKY and the SHR rats (see Fig. 4). These four proteins are considered as being genetic markers for hypertension. A more detailed picture of one of these proteins # 847 is shown in Fig. 6. Full statistical analysis revealed a total of 29 proteins whose expression was strongly correlated to the blood pressure of the individual (Fig. 5). These proteins were selected on the basis that either 1) there was a greater than 95% probability of correlation between blood pressure with protein expression in both the F2 and parental generations; or 2) that the protein showed greater than 95% probability of correlation in either the F2 or parental generation and a greater than 90% probability of correlation in the other generation.

An attempt was then made to further identify and characterize the hypertension markers. The first approach to protein identification was to use microsequencing and the second was

to use matrix assisted laser desorption time of flight mass spectrometry (MALDI tof MS). Using microsequencing it has been possible to find amino acid sequence information for four of these markers. The results obtained are quite outstanding since - as will be apparent from Fig. 7 - the marker #325 shows a very large sequence homology with an already known protein, Angiotensin-I converting enzyme (also known as ACE-1). The sequence homology now found is located at the N-terminal part of ACE-1 which is quite conserved among different species and from which the expression is regulated. Subsequent studies using MALDI tof MS have been used to characterise the 29 proteins. To date, spectra have been obtained for 2 proteins. These proteins are listed in Table 1 and the individual spectra are shown in Figures 12 - 13.

The present finding is interesting as either wrong or too large/too little synthesis of ACE-1 has been associated with a very large number of cardiac/vascular diseases, and in particular with the development of hypertension. The reason for this interconnection may be seen in Fig. 8 where ACE-1 first of all takes an active part in synthesizing a vasopressor and, secondly, contributes to the destruction of a vasodilator (bradykinin). Thus, ACE-1 has a double negative influence on hypertension.

Moreover, another group of researchers has been able to show that if monoclonal antibodies to ACE-1 are synthesized and then injected into hypertensive animals, nothing will happen! (Danilov et al., 1991). On the contrary, if polyclonal antibodies are synthesized (which might, e.g., recognize "the present form of ACE-1"), the animals will die very quickly (Danilov et al., 1991) (see Fig. 9)

The especially interesting feature of the present findings is that it is 100% certain that the protein #325 in question is not identical with ACE-1 [the amino acid sequence is different, molecular weight and pI are not the same (neither are they identical with degradation products of ACE-1)].



The present inventors are therefore of the opinion that they have identified at least one protein/gene which may take over the regulation of the blood pressure in hypertensive individuals, e.g. as a replacement of ACE-1, but which cannot be regulated in the same way. Furthermore the inventors have identified a number of other proteins which are involved, directly or indirectly, in the process leading to or sustaining hypertension. The further analysis of the protein # 325 and its possible relationship to the 52 kD protein described by Müns et al. is outlined in Fig. 10.

Further, some of the proteins identified by MALDI tof MS have been related to human diseases and thus their function have been shown (in other contexts) to be crucial to the health of the individual, but none of the proteins have been associated to hypertension.

The present findings may be used in connection with the diagnosis of a genetic predisposition for hypertension which will enable the individual to take appropriate actions with respect to his or her life style in order to minimize the potential adverse effects of the genetic predisposition.

Also, it is believed that the present invention will be useful in the treatment of hypertension which is a disease for which there is currently no effective treatment. As an example, it is within the scope of the present invention to replace a defective gene in a patient with a genetically engineered gene wherein the defect is cured and insert it into the somatic cells of the patient in order to correct a defective gene which is at least partly responsible for the hypertension. Such gene transfer can take place e.g. by use of a retroviral vector or by use of DNA liposomes. In another aspect, the present invention thus relates to a test animal having a nucleotide sequence of the invention which may be used for an effective screening of novel medicaments for the treatment of hypertension.

## DETAILED DISCLOSURE OF THE INVENTION

In one aspect, the invention relates to a method using proteome analysis involving the separation of proteins obtained from biological materials by 2DGE; the computer analysis of  
5 the resulting images; the statistical analysis of the data to ~~select the proteins which play a role, directly or indirectly~~ in the studied biological problem; and the identification and characterisation of the selected proteins by microsequencing or by mass spectrometry. In the present case the invention  
10 relates to a method for determining at least one marker protein which is indicative of a high likelihood of having hypertension or a genetic predisposition for having hypertension, the method comprising performing a 2D gel electrophoresis of a sample from at least one mammal having  
15 hypertension and a 2D gel electrophoresis of a sample from at least one mammal which does not have hypertension, analyzing and comparing the two 2D gel electrophoresis patterns by computer and determining at least one marker protein which is expressed in a significantly different amount in the hyper-  
20 tensive mammal. It should be noted that the detection of any combination of more than one of the markers would be expected to make the analysis an even more reliable indicator for hypertension. Combinations of two markers would thus be preferred and three or more markers would be strongly pre-  
25 ferred. In Example 1 this approach is exemplified with reference to rats. It is evident that it will be possible to perform similar experiments with respect to other mammals, including human beings.

Furthermore, it is likely that at least some of the marker  
30 proteins are conserved within species and thus that the markers proteins found by use of the hypertensive rats will also be useful in diagnosis of hypertension in other species including human beings. In a further aspect, the invention thus relates to a method of detecting for an individual  
35 mammal a high likelihood of having hypertension or a genetic predisposition for having hypertension, the method comprising

determining in a biological sample from the individual mammal the presence of at least one marker protein which is indicative of a high likelihood of having hypertension or a genetic predisposition for having hypertension. The mammal may e.g.  
 5 be a rat, mouse, rabbit, human or bovine species.

According to the invention, any biological sample that contains or is suspected of containing at least one marker of the invention can be analyzed. Examples of such biological samples include, but are not limited to, blood, serum, plasma, tissue biopsy, organ biopsy, synovial fluid, urine, bile  
 10 fluid, cerebrospinal fluid, saliva, mucosal secretion, effusion, and sweat. It should be recognized that one or more of the markers may be present in soluble or solubilized form in such samples, or may be present in cells isolated with such  
 15 samples. In the latter instance, the markers may be biosynthesized after isolation of the sample, thus providing an opportunity for biosynthetic labelling.

In the method according to the invention, the marker protein  
 20 is selected from the group consisting of the following proteins listed in Table 1 below:

Table 1

	Marker protein	MW	pI	Fig. No.*	Regulation
25	27	156,000	5.85	12	-
	189	104,000	4.76		-
	126	93,000	5.91		+
	412	66,700	4.62		-
	355	64,400	5.44		+
30	352	57,500	5.73		+
	325	57,500	6.39		-
	452	53,400	5.90		+
	509	50,100	4.77		-
	870	47,600	5.25		-
35	1464	46,300	6.11		+

	603	46,000	4.79	-
	667	39,900	5.02	-
	657	38,900	5.39	-
	686	37,300	4.71	+
5	662	35,800	5.33	-
	711	35,100	5.49	-
	1180	33,600	5.28	-
	746	29,300	6.27	-
	750	28,900	6.06	-
10	777	27,300	4.62	-
	785	25,600	4.50	13 -
	803	24,600	5.28	-
	847	17,400	4.49	-
	1031	17,300	5.70	-
15	1033	17,200	4.93	-
	1032	16,300	5.25	-
	862	14,900	4.65	-
	864	14,500	4.73	-

and including any modifications and derivatives of these  
 20 marker proteins, e.g. homologous proteins from other species,  
 wherein pI is the isoelectric point of the marker protein as  
 determined by isoelectric focusing, and the molecular weight  
 (MW) is determined on a polyacrylamide gel. The modified  
 forms of a native protein may for example be glycosylated,  
 25 phosphorylated, acetylated, methylated, or lipidified forms.

Notes to table:

\*) Where applicable the mass spectrum obtained by MALDI tof  
 MS analysis of tryptic peptide fragments of the protein is  
 shown in the figure.

30 The molecular weights of the peptides which gave rise to the  
 peaks seen in the mass spectrum are calculated. All protein  
 sequence information which have been entered in all publicly  
 available databases is accessed and each protein is theoret-  
 ically "digested" with trypsin to generate individual mole-

cular weight list for each protein. The values observed are then compared with those calculated from the databases. The actual protein is considered to be unequivocally identified when sufficient molecular weights match (between 4 and 8).

- 5 Since not all proteins are described in the databases that exist at present, it is quite possible to have a spectrum which does not match any known protein. Even so this spectrum is considered to be an unequivocal identification of the protein.
- 10 Finally, if the protein sequence is sufficiently well conserved between species then this spectrum can also identify proteins across species barriers. Therefore the identified equivalent human proteins have also been inserted into Table 2 below. Corresponding proteins from other species were also
- 15 identified but are not inserted for clarity.

As examples, the following markers have been further identified as outlined above:

Table 2

Marker protein	Swissprot Accession Number and Identity
27	Q03410 SCP1_RAT SYNAPTONEMAL COMPLEX PROTEIN 1 (SCP1 PROTEIN)
785	Q64686 CAG7_RAT Alpha-N-acetylgalactosaminide Alpha-2,6-sialyltransferase (EC 2.4.99.-) (ST6GALNACIII) (STY).
25	Q11203 CAG6_HUMAN
	Q92185 Q93064 CAG8_HUMAN
	Q92186 Q92470 CAG9_HUMAN

- 30 In a similar manner and as described in detail in the examples, MALDI tof MS data regarding the other protein markers will be obtained.

In the present specification and claims, the term "peptide" comprises a) short peptides with a length of at least two amino acid residues and at most 10 amino acid residues; b) oligopeptides (11-100 amino acid residues) as well as  
5 c) proteins (greater than 100 amino acid residues), the functional entity comprising at least one peptide, oligopeptide, or polypeptide. Any of the above may be chemically modified by being glycosylated, by being lipidated, or by comprising prosthetic groups. The definition of peptides also  
10 comprises native forms of peptides/proteins in animals including humans as well as recombinant proteins or peptides in any type of expression vectors transforming any kind of host, and also chemically synthesized peptides.

One specific embodiment of the invention relates to a peptide  
15 which is not present or present in a low amount in 2D gel electrophoresis of proteins from resistance artery biopsies from hypertensive rats and which is present in a significantly higher amount in 2D gel electrophoresis of proteins from resistance artery biopsies from normotensive rats. This  
20 embodiment comprises the marker proteins marked with "-" in Table 1.

Another embodiment of the invention relates to a peptide which is present in 2D gel electrophoresis of proteins from resistance artery biopsies from hypertensive rats and which  
25 is not present or present in a significantly lower amount in 2D gel electrophoresis of proteins from resistance artery biopsies from normotensive rats. This embodiment comprises the marker proteins marked with "+" in Table 1.

In particular, the invention relates to a peptide which has a  
30 molecular weight of about 57.5 kD and an isoelectric point of about 6.39, the peptide comprising the following partial amino acid sequence Arg Ser Ala Pro Gly Leu Asn Ser Gly X X Pro Ala Glu Glu Val (SEQ ID NO:1). Further, the invention relates to a peptide which has a molecular weight of about  
35 57.5 kD and an isoelectric point of about 6.39, the peptide

comprising the following partial amino acid sequence  
(Met) Leu His Glu Leu Glu Lys Ala Tyr (Arg) Phe (Lys) (SEQ ID NO:2). Moreover, the invention relates to peptides which contain both partial sequences.

- 5 A third embodiment of the invention is a peptide which has a molecular weight of about 17.4 kD and an isoelectric point of about 4.49, the peptide comprising the following partial amino acid sequence X Pro Thr Glu Ala Ala X (SEQ ID NO:3).

- 10 A fourth embodiment of the invention relates to a peptide which has a molecular weight of about 14.9 kD and an isoelectric point of about 4.65, the peptide comprising the following partial amino acid sequence Met Gln Ala Glu Met Ser Pro Ala Phe X Ser Tyr X X Gln (SEQ ID NO:4).

- 15 The invention further relates to a peptide which has a degree of homology of at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95%, with an amino acid sequence selected from the group consisting of sequences SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4 irrespective of any modifications of said amino acids.
- 20 When determining homology, modified amino acids such as phosphorylated, acetylated, amidated, methylated, glycosylated or lipidated derivatives of an amino acid should thus be considered to be the same as the amino acid without any such modification. Such peptides may be derived from similar proteins from other species, e.g. other mammals such as mouse,
- 25 rabbit, guinea pig, pig, cow or human or may be entirely or predominantly of synthetic origin.

- By the term "homology" is thus meant the overall similarity in the sequence of amino acids in the match with respect to
- 30 identity and position of the amino acids of the peptides.

If longer amino acid sequences are compared, the degree of homology required may be lower, such as when a consecutive string of 6, 7, 8, 9 or 10 amino acids are selected from the

amino acid sequences shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4. Then the degree of homology may be at least 62.5%, such as at least 66%, or at least 70%. Under certain circumstances, it is advantageous that the degree of  
5 homology is even higher such as at least 83% or at least 90%.

In particular, the invention relates to a peptide selected from the group consisting of

	Pro Gly Leu Asn Ser Gly X X Pro Ala Glu Glu Val (SEQ ID NO: 5)
	Pro Gly Leu Gln Ser Gly X X Pro Ala Glu Glu Val (SEQ ID NO: 6)
10	Pro Gly Leu Asn Pro Gly X X Pro Ala Glu Glu Val (SEQ ID NO: 7)
	Pro Gly Leu Gln Pro Gly X X Pro Ala Glu Glu Val (SEQ ID NO: 8)
	Pro Gly Leu Asn Ser Gly X X Ser Ala Glu Glu Val (SEQ ID NO: 9)
	Pro Gly Leu Gln Ser Gly X X Ser Ala Glu Glu Val (SEQ ID NO: 10)
	Pro Gly Leu Asn Pro Gly X X Ser Ala Glu Glu Val (SEQ ID NO: 11)
15	Pro Gly Leu Gln Pro Gly X X Ser Ala Glu Glu Val (SEQ ID NO: 12)
	Pro Gly Leu Asn Ser Gly X X Pro Ala Asp Glu Val (SEQ ID NO: 13)
	Pro Gly Leu Gln Ser Gly X X Pro Ala Asp Glu Val (SEQ ID NO: 14)
	Pro Gly Leu Asn Pro Gly X X Pro Ala Asp Glu Val (SEQ ID NO: 15)
	Pro Gly Leu Gln Pro Gly X X Pro Ala Asp Glu Val (SEQ ID NO: 16)
20	Pro Gly Leu Asn Ser Gly X X Ser Ala Asp Glu Val (SEQ ID NO: 17)
	Pro Gly Leu Gln Ser Gly X X Ser Ala Asp Glu Val (SEQ ID NO: 18)
	Pro Gly Leu Asn Pro Gly X X Ser Ala Asp Glu Val (SEQ ID NO: 19)
	Pro Gly Leu Gln Pro Gly X X Ser Ala Asp Glu Val (SEQ ID NO: 20)
	Pro Gly Leu Asn Ser Gly X X Pro Ala Glu Glu Ala (SEQ ID NO: 21)
25	Pro Gly Leu Gln Ser Gly X X Pro Ala Glu Glu Ala (SEQ ID NO: 22)
	Pro Gly Leu Asn Pro Gly X X Pro Ala Glu Glu Ala (SEQ ID NO: 23)
	Pro Gly Leu Gln Pro Gly X X Pro Ala Glu Glu Ala (SEQ ID NO: 24)
	Pro Gly Leu Asn Ser Gly X X Ser Ala Glu Glu Ala (SEQ ID NO: 25)
	Pro Gly Leu Gln Ser Gly X X Ser Ala Glu Glu Ala (SEQ ID NO: 26)
30	Pro Gly Leu Asn Pro Gly X X Ser Ala Glu Glu Ala (SEQ ID NO: 27)
	Pro Gly Leu Gln Pro Gly X X Ser Ala Glu Glu Ala (SEQ ID NO: 28)
	Pro Gly Leu Asn Ser Gly X X Pro Ala Asp Glu Ala (SEQ ID NO: 29)
	Pro Gly Leu Asn Pro Gly X X Pro Ala Asp Glu Ala (SEQ ID NO: 30)
	Pro Gly Leu Gln Pro Gly X X Pro Ala Asp Glu Ala (SEQ ID NO: 31)
35	Pro Gly Leu Gln Ser Gly X X Pro Ala Asp Glu Ala (SEQ ID NO: 32)
	Pro Gly Leu Asn Ser Gly X X Ser Ala Asp Glu Ala (SEQ ID NO: 33)



Pro Gly Leu Gln Ser Gly X X Ser Ala Asp Glu Ala (SEQ ID NO: 34)  
Pro Gly Leu Asn Pro Gly X X Ser Ala Asp Glu Ala (SEQ ID NO: 35)  
Pro Gly Leu Gln Pro Gly X X Ser Ala Asp Glu Ala (SEQ ID NO: 36)

5 In another embodiment, proteins which are capable, after  
trypsin digestion, of producing mass spectra peaks when  
analysed by mass spectrometry which could correspond to at  
least 3 or more preferably 6 of the peaks detected on the  
spectra from individual proteins (shown in Figures 12 - 13)  
are considered as homologous to or identical with the 29  
10 hypertension marker proteins described in this patent appli-  
cation and thus within the scope of the present invention.  
Some of these mass spectra, when compared to the database  
information publicly available (in manner described in the  
note to Table 1) corresponded to known proteins. Even though  
15 they are known, none had previously been associated with a  
potential role in the development of hypertension and so this  
association is novel. These proteins are listed in Table 1  
and the accession numbers for the human and rat proteins are  
given. Thus, the entire sequence of these proteins (including  
20 the corresponding proteins from other species) showing levels  
of homology as defined above is thus included in the scope of  
this invention with respect to their relationship to hyper-  
tension in a manner similar to that of the sequences obtained  
by microsequencing.

25 Due to the degeneracy of nucleotide coding sequences, other  
DNA sequences which encode substantially the same amino acid  
sequence as a gene encoding a marker protein, i.e. a marker  
gene, may be used in the practice of the present invention.  
These include, but are not limited to, allelic genes, homolo-  
30 gous genes from other species, and nucleotide sequences  
comprising all or portions of marker genes which are altered  
by the substitution of different codons that encode the same  
amino acid residue within the sequence, thus producing a  
silent change. Likewise, the marker derivatives of the in-  
35 vention include, but are not limited to, those containing, as  
a primary amino acid sequence, all or part of the amino acid

sequence of a marker protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or  
5 more amino acid residues within the sequence can be substituted by another amino acid of similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino  
10 acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged  
15 (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight determined by polyacrylamide gel electrophoresis. For situations where one charged  
20 amino acid is exchanged with another (similarly or differently charged, or uncharged) amino acid, the changes in pI will be more readily detectable in small proteins whose pI falls within the interval between the pKas of the groups involved. Such a change may be imperceptible for large proteins.

25 The invention comprises any peptide which comprises a sequence which differs from an amino acid sequence as defined above in that at least one amino acid has been deleted, substituted or modified or at least one additional amino acid has been inserted so as to result in an amino acid sequence  
30 which encodes a peptide which is a hypertension marker protein useful in the method of the invention.

Nucleic acid fragments comprising a nucleotide sequence which codes for a peptide according to the invention as well as nucleic acid fragments which hybridize with these nucleic  
35 acid fragments or a part thereof under stringent hybridization conditions, e.g. 5 mM monovalent ions (0.1xSSC), neutral

pH and 65°C are important aspects of the invention. The term "highly stringent", when used in conjunction with hybridization conditions, is as defined in the art, i.e. 5-10°C under the melting point  $T_m$ , cf. Sambrook et al, 1989, pages 11.45-5 11.49.

By the term "nucleic acid" is meant a polynucleotide of high molecular weight which can occur as either DNA or RNA and may be either single-stranded or double-stranded.

To the extent the amino acid sequences of the marker proteins 10 comprised by the present invention are known, it is possible to synthesize DNA or RNA probes which may be used for:

- i) direct detection of DNA and RNA expressing said marker proteins on a fixed or frozen tissue section using, e.g., chromogenous, chemiluminescent or 15 immunofluorescent techniques;
- ii) polymerase chain reaction (PCR); and
- iii) locating the part or all of the gene, isogene, pseudogene or other related genes either in cDNA libraries, genomic libraries or other collections of 20 genetic material from either the host or other animals, including man.

Within the scope of the present invention is the use of nucleic acid fragment(s) for detecting the presence of a peptide according to invention. In particular, nucleic acid 25 fragments which code for a peptide of the invention and fragments which hybridize with nucleic acid fragments coding for a peptide of the invention or a part thereof under stringent or highly stringent hybridization conditions are considered useful in a PCR-DNA analysis method of detecting whether an individual has a high likelihood of having hypertension or a genetic predisposition therefor. The length of the 30 primer is preferably at least 12, more preferably at least 13 or 15 and often about 18 nucleotides.

The difference in sequences which is decisive in this type of PCR according to the invention can be identified e.g. from Fig. 7 and confirmed by simple experiments.

Especially, the first primer is a DNA sequence of 15-25 nucleotides which differs from any subsequence of the proteins ~~from the other species in at least 4 nucleotides per 20~~ nucleotides of the primer, preferably the differences being in 5 nucleotides per 20 nucleotides.

When differences are present in 4 or more nucleotides per 20 nucleotides of a primer a minimum of cross-reaction between the primer and a protein from another species is possible irrespective of the stringency hybridization conditions.

In order to detect the presence of a marker protein of the invention without knowing the exact marker protein searched for beforehand, it is preferred to use a combination of different primers, comprising, for each of marker proteins to be detected, a type of primer unique to the protein and differing from any subsequences of similar normal proteins from the same or other species in at least 4 nucleotides per 20 nucleotides of primers. Preferably, for specific detection the PCR kit should include a set of 2 primers for each marker peptide to be detected, e.g. a kit having 3 sets of 2 primers in appropriate amounts together with other PCR reagents.

The PCR analysis is performed in accordance with usual PCR technique such as described in the literature. Thus, for the detection, the primer may be labelled, such as with radioactive labels, fluorescent dyes, and biotin, or labelled nucleotide triphosphates (e.g. labelled with thymidine) can be included in the PCR reaction to label the PCR amplification product.

The PCR primers used according to the invention may be prepared using well-known methods. Thus, they may be prepared by oligonucleotide synthesis, or they may be prepared by frag-

mentation of a larger nucleotide sequence using suitable restriction enzymes. The labelling of the primers can be performed by methods well-known per se.

As is conventional, the PCR reagents can be included in  
5 suitable PCR kits.

Using the PCR analysis method or kit as described above, it is possible to diagnose hypertension or a genetic predisposition therefor in mammals, including humans, independent of the specific marker protein.

10 In another aspect, the invention relates to a binding means which specifically binds to a peptide shown in Table 1 or a peptide or nucleic acid fragment as described above. In particular, the invention relates to an antibody which specifically binds to a peptide of the invention or an antigen-  
15 binding fragment thereof, i.e. a polyclonal antibody, a monoclonal antibody, chimeric antibody, single chain antibody fragment, Fab and Fab' fragments, and an Fab expression library.

It is contemplated that both monoclonal and polyclonal antibodies  
20 will be useful in providing the basis for one or more assays to detect peptides and marker proteins. Antibodies which are directed against epitopes that are specific for the marker proteins will be most useful as cross reaction will be minimized therewith.

25 Preferred immunoassays are contemplated as including various types of enzyme linked immunoassays (ELISAS), immunoblot techniques, and the like, known in the art. However, it is readily appreciated that utility is not limited to such assays, and useful embodiments include RIAs and other non-  
30 zyme linked antibody binding assays or procedures.

An important embodiment of the invention is a test kit for detecting whether an individual mammal has a high likelihood

of having hypertension or a genetic predisposition for having hypertension, comprising:

- a) binding means which specifically binds to at least one marker protein shown in Table 1 or at least one peptide or  
5 nucleic acid fragment described above,
- 
- b) means for detecting binding, if any, or the level of binding of the binding means to at least one of the marker proteins or at least one of the peptides or nucleic acid fragments, and
- 10 c) means for correlating whether binding, if any, or the level of binding, to said binding means is indicative of the individual mammal having a significantly higher likelihood of having hypertension or a genetic predisposition for having hypertension.
- 15 Although the testing for and the detection of one marker protein may be sufficient, it is likely that testing for and the detection of more than one marker protein (or its nucleotide sequence (e.g. DNA, cDNA or RNA) will be much more valuable for the identification and characterisation of  
20 hypertension.

Another important embodiment relates to a test kit for detecting whether an individual mammal has a high likelihood of having hypertension or a genetic predisposition for having hypertension, comprising:

- 25 a) a nucleic acid according to the invention,
- b) means for detecting binding, if any, or the level of binding, to a nucleic acid as described above, and
- c) means for correlating whether binding, if any, or the level of binding, to said nucleic acid is indicative of the  
30 individual mammal having a significantly higher likelihood of

having hypertension or a genetic predisposition for having hypertension.

Likewise, a method of determining the presence of a marker protein in a mammal, including a human being, or in a sample, comprising administering a binding means of the invention to the mammal or incubating the sample with the binding means, and detecting the presence of bound marker protein resulting from the administration or incubation, forms part of the invention.

- 10 In a further aspect, the invention relates to a method for determining the effect of a substance, the method comprising using a mammal which has been established to be an individual having a high likelihood of having hypertension or a genetic predisposition for having hypertension by use of the method  
15 of the invention, the method comprising administering the substance to the individual and determining the effect of the substance e.g. on the blood pressure of the individual.

Within the scope of the invention is a pharmaceutical composition which comprises a substance which is capable of regulating the expression of a nucleic acid as defined above, at  
20 at least one marker protein shown in Table 1 or a peptide as defined above or the activity of at least one marker protein shown in Table 1 or a peptide and/or a nucleic acid as defined above. In particular, the invention comprises a pharmaceutical composition comprising at least one marker protein  
25 shown in Table 1 or a peptide, a nucleic acid fragment or a binding means as defined above and the use of a such nucleic acid fragments for the treatment or prophylaxis of hypertension (antisense and sense therapy).

- 30 The invention is further illustrated in the following, non-limiting Examples.

## EXAMPLE 1

## IDENTIFICATION OF MARKERS OF HYPERTENSION

In this example, specific proteinaceous markers indicating hypertension are identified. The identified proteins are synthesized in significantly different amounts in hypertensive F2-rats relative to their expression in normotensive F2-rats. These findings provide a new basis for understanding the genetic basis of hypertension, and not least will enable an earlier detection of hypertension. Accordingly, treatment to avoid hypertension and associated organ damage may be intensified at an earlier stage.

The strategy followed in obtaining the data is outlined in Fig. 11.

Resistance arteries, approximately 2 mm, were collected by microsurgery from the mesenteric bed, washed in Hank's buffer, and placed into 100  $\mu$ l of labelling medium [DMEM without methionine, containing 10% dialysed human AB serum and 1 mCi/ml [ $^{35}$ S]-methionine (Amersham SJ204)] and incubated for 20 hours at 37°C in a 5% CO<sub>2</sub>/95% air humidified atmosphere. The biopsies were homogenized on ice in microhomogenizers using 100  $\mu$ l of RNase/DNase buffer containing 25  $\mu$ g/ml RNase A (Cooper, Catalog No. LS05650), 25  $\mu$ g/ml DNase I (Cooper, Catalog No. LS06330), 30 mM NaCl, 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 20 mM Tris-HCl, pH 8.5, and left to digest for 30 minutes at 4°C. They were then snap frozen in liquid nitrogen and freeze dried before solubilization in loading buffer [9.5 M urea, 2% w/v NONIDET-P40® (NP-40), 2% w/v AMPHOLINES® pH range 7-9 (Pharmacia), 5% w/v 2-mercaptoethanol]. Polyacrylamide gels (3.5%, 185 x 1.55 mm, 8 M urea, 2% NP-40) were prepared containing: for the IEF gels 6% AMPHOLINES® pH range 3.5-10, 2% AMPHOLINES® 5-7, and 2% SERVALYTES® 5-7 (Serva); and for the NEPHGE gels 3.3% AMPHOLINES® 7-9, 3.3% AMPHOLINES® 8-9.5, and 0.5% AMPHOLINES® 3.5-10. In each case, the actual mixture of ampholytes was calibrated for each batch. Phosphoric acid



(H<sub>3</sub>PO<sub>4</sub>; 10 mM) was used as the anode buffer and degassed NaOH (20 mM) was used as the cathode buffer. IEF gels were pre-focused at 1200 V, 133  $\mu$ A (limiting values) per tube until the limiting voltage was reached. Half a million TCA precipitable  
5 counts were applied to both the IEF and NEPHGE gels and samples were overlayed with 8 M urea, 0.8% AMPHOLINES® (pH 5-7), and 0.2% AMPHOLINES® (pH 3.5-10). Electrophoresis was carried out at 1200 V, 133  $\mu$ A (limiting) per gel: for 18 hours for IEF gels; and for 4 hours after the gels reached  
10 the limiting voltage for the NEPHGE gels. The gels were then extruded using compressed air and equilibrated for 6 minutes before being frozen at -70°C.

When ready for further analysis, the gels were quickly thawed in a water bath (80°C), incubated for 2 minutes at room  
15 temperature, loaded and run on 12.5% polyacrylamide gels (200 x 200 mm, 1 mm) at 20°C. The second dimension gels were fixed for 45 minutes in 45% methanol, 7.5% acetic acid, treated for 45 minutes with AMPLIFY® (Amersham) to enhance fluorographic detection, dried, and exposed to X-ray film (AGFA Curix RP2)  
20 at -70°C for 5 days. The developed images were compared either visually or with an image processing computer (BioImage programme version 4.5 M, Millipore).

Images were captured by either scanning the autoradiographs, fluorographs or dried silver stained gels using a Truvel  
25 scanner (Bio Image) or by importing the TIFF files from the fluorescence imager or phosphor imager (AGFA ADC). The images were edited and matched using the Bio Image software (version 6.1) on a Sun Ultrasparc workstation.

Spot numbers and their corresponding integrated optical  
30 densities (expressed as a percentage of the total blackening of the image attributed to proteins, IOD%) were transferred to a spreadsheet program for statistical analysis.

After exposure, the developed X-ray film was positioned over the dried gel using the crosses and used to locate the pro-

teins of interest. A stencil-mask was made to relocate these proteins once the X-ray film was removed and the proteins were cut directly from the gel, taking as little backing filter paper with the spot as possible.

- 5 For mass spectrometry, in gel reduction, alkylation and  
tryptic digestion were similar to published procedures, see  
e.g. Kussmann et al., 1997. Protein spots were cut directly  
from the gels. The gel pieces were washed with 60% 100 mM  
10  $\text{NH}_4\text{HCO}_3$  and 40% acetonitrile (this also removed some of the  
coomassie blue stain prior to mass spectrometry). The re-  
remaining white filter paper was removed from the back of the  
gel with tweezers. The gel piece was then dried in a vacuum  
centrifuge until it became white on the surface and then the  
gel was allowed to reswell in 50 mM  $\text{NH}_4\text{HCO}_3$ , 5 mM  $\text{CaCl}_2$ , 12.5  
15 ng/ $\mu\text{l}$  trypsin (Boehringer Mannheim, sequencing grade) at 4°C  
for 45 mins. The supernatant was then replaced with 5-10  $\mu\text{l}$   
of the same buffer lacking trypsin and left to digest over-  
night at 37°C.

- Mass spectra were recorded on a Bruker Reflex (Bruker-Fran-  
20 zen, Bremen, Germany) or a Voyager Elite (PerSeptive Bio-  
systems, Framingham, Massachusetts, USA) mass spectrometer,  
both operated in delayed extraction reflector mode. Samples  
were prepared using cyano-4-hydroxy cinnamic acid as matrix.  
When appropriate, nitrocellulose was mixed with the matrix.  
25 The criteria for choice of matrix and the sample preparation  
procedures are described in detail elsewhere [10].

- Nano electrospray mass spectra were obtained on a Finnigan TSQ  
70 triple quadrupole mass spectrometer (Finnigan Corporation,  
San Jose, California, USA) or a Bruker Esquire ion trap mass  
30 spectrometer (Bruker-Franzen, Bremen, Germany) both equipped  
with nano electrospray sources. Sample preparation was per-  
formed as described above.

All ampholines used in the foregoing experiments were obtai-  
ned from Pharmacia, with the exception of the SERVALYTES®

ampholines obtained from Serva. One example of the resulting 2D Gel of [<sup>35</sup>S]-methionine labelled resistance artery is shown in Fig. 5. The locations of all the hypertension marker proteins are shown by their internal computerised reference numbers. Comparison of such patterns clearly illustrates, by differences in protein spot intensity, a number of very significant changes in protein expression: proteins that are expressed in WKY rats or normotensive F2-rats are expressed at non-detectable or low detectable levels in SHR rats and hypertensive F2-rats or vice versa. As such, the proteins marked in Figure 5 and listed in Table 1 could be used as markers for hypertension.

An example for one of the hypertension markers # 847 is shown in Fig. 6. Four examples of the resulting 2D Gel of [<sup>35</sup>S]-methionine labelled resistance artery are shown. Comparison of these patterns clearly illustrates, by differences in protein spot intensity, a number of very significant changes in protein expression: proteins that are expressed in WKY rats or normotensive F2-rats are expressed at non-detectable or low detectable levels in SHR rats and hypertensive F2-rats (indicated by arrows in Fig. 6).

It was possible to detect 29 markers for hypertension which are summarized in Table 1. The level of expression of the markers can be analyzed by computerized image analysis, thus allowing quantitative analysis. Quantitation of the films demonstrated that some of the marker proteins were overexpressed and some were expressed in a lower amount than in normotensive rats.

Further, the hypothesis was tested as indicated in Fig. 4 from which it appears that the data indicate a strong correlation for 847, 803, 325 and 862 with respect to blood pressure.

As noted in the following Example, partial amino acid sequence information has been obtained from three or four proteins.

## EXAMPLE 2

### 5 DETERMINATION OF PARTIAL AMINO ACID SEQUENCES

A number of marker proteins identified on the IEF gels were analyzed further by microsequencing. The proteins were electroeluted using a 0.1% SDS, 27.5 mM Tris-HCl, 95.9 mM glycine, pH 8.3 buffer and recovered on PVDF membrane. The membrane was washed and saturated with 0.2% polyvinylpyrrolidone. The membrane-associated protein was the trypsin digested with 0.5  $\mu$ g trypsin for 24 hours at 37°C in 100  $\mu$ l of 100 mM Tris-HCl, pH 8.5, 10% acetonitrile. The peptides were separated by reverse phase-HPLC using a C-18 column with a trifluoroacetic acid-acetonitrile gradient. Peaks were detected at 214 nm and manually collected and treated with POLYBRENE® (Applied Biosystems). Sequencing of the peptides was carried out in a pulsed-liquid phase sequencer (Applied Biosystems, model 477A) equipped with an on-line phenylthiohydantoin amino acid derivative analyzer (Applied Biosystems, model 120A). The information obtained is reported as sequences SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4 above.

After the partial sequence information was obtained, the sequences were searched for homology or similarity to known proteins. The programme GCG (Genetics Computer Group, Wisconsin) was used to search the SWISS-PROT protein sequence database (40,292 sequences, 14,147,368 symbols) with the FASTA subroutine, and using the EMBL genbank (257,716 sequences, 263,275,079 symbols) using the subroutine tFASTA so that the amino acid sequences were translated to base sequences and these were used to search all entered sequences using a word size of 2.

The first partial sequence (SEQ ID NO:1) for protein 305 showed high similarity with ACE-1. A second partial sequence from this spot (SEQ ID NO:2) did not demonstrated any significant homology or similarity with ACE-1. Both proteins  
5 appear to be novel proteins, i.e., proteins that have not been sequenced previously. The sequence data therefore suggest that at least one of the polypeptides which have been detected corresponds to a new variant of ACE, having a different amino acid sequence, a different size, and a different  
10 isoelectric point.

Circumstantial evidence for the importance of this or these molecules can be obtained by careful examination of the literature.

Müns et al. (1993) have shown that monoclonal antibodies  
15 raised against ACE detect one single band of a molecular weight of 175 kD on Western blotting, corresponding to the correct size of ACE. Polyclonal antibodies raised against ACE detect three bands at 175, 52 and 47 kD, where the band at 52 kD was the strongest. Müns et al. postulated that the  
20 bands at 52 kD and at 47 kD were degradation products of the 175 kD ACE.

In a totally separate research project, Danilov et al. (1991) produced monoclonal and polyclonal antibodies in much the same way as Müns et al. and showed that injection of the  
25 monoclonal antibodies into mice had no effect on the mice, whereas, when the polyclonal antibodies were injected, this killed the mice. However, the proteins recognized by these antibodies were not characterized.

The interpretation of these two separate pieces of evidence  
30 is that inactivation (by complexing with the monoclonal antibody) of the 175 kD ACE is not lethal to the mice, whereas inactivation of the 175, 52 and 47 kD proteins (by complexing with the polyclonal antibodies) is lethal. This would therefore suggest that these three proteins are essential to

the animal - i.e. are more important than the ACE alone. Due to the inaccuracies known to persons skilled in the art who are to measure molecular weights by gel electrophoresis, it is suggested that the 52 and 47 kD proteins correspond to some of the proteins encountered in connection with the present findings. The fact that the present peptide (SEQ ID NO:1) shares some homology (and therefore would be recognized by the antibody) and has a similar size to the 52 kD protein strongly suggests that these are one and the same protein. This comparison is supportive for the role that there is an extra ACE-like protein and most importantly, that this protein has a role which is at least as important as that of ACE.

#### LEGEND TO FIGURES

Fig. 1 shows the genetic relationships between WKY and SHR rats. WKY and SHR rats were crossed, yielding an F1 generation. These F1 rats were then crossed with each other in order to obtain an F2 generation, and the rats of this generation were used for the studies. As expected, this F2 generation showed a larger range of the blood pressures of the rats than the parental generation.

Fig. 2 shows the blood pressure distribution among WKY and SHR rats and the F2 rats. The mean blood pressure (mm Hg) is shown on the abscissa, and the different groups are shown on the ordinate.

Fig. 3 shows raw data with respect to protein expression in normal and hypertensive rats. The numbers in the column headings relate to the individual rats. The abbreviation AVG stands for average; STDS stands for standard deviation; and t-value is from the Student's t-test.

Fig. 4 shows the 10 most significant correlations (by Student's t-test) of blood pressure with protein expression in the F2 generation (middle panel). The same 10 proteins are

selected from the parental generation (right-hand panel) and the Figure shows that four of these (proteins 847, 803, 325 and 862) were found to be highly correlated also in the parental generation. %IOD in this case is the average %IOD  
5 for each protein from the whole group. Std is the standard deviation for each protein from the said group. P is the normal probability value for the comparison between the F2 and the parental generation for each protein. NS means not significant.

10 Fig. 5 shows the two-dimensional gel of [ $^{35}\text{S}$ ]-methionine labelled resistance arteries. All the proteins whose expression is significantly correlated to blood pressure are indicated with circles with a cross (if the protein is downregulated in the hypertensive relative to the normotensive) or  
15 without a cross (if the protein is upregulated in the hypertensive relative to the normotensive).

Fig. 6 shows a detailed picture of protein # 847 from the regions of the gels from the normotensive and hypertensive rats from the F2 and the parental generations.

20 Fig. 7 shows the amino acid sequences of the hypertension marker #325 compared with the sequence of ACE from various species. Note that some amino acids are completely conserved and some are less well conserved.

Fig. 8 shows the functions of the angiotensin-converting  
25 enzyme (ACE). Angiotensinogen is cut by the protease renin to the inactive peptide angiotensin I. ACE cuts angiotensin I to angiotensin II (which is a potent vasopressor). At the same time, ACE can inactivate the active vasodilator bradykinin by proteolysis and so ACE has a double effect.

30 Fig. 9 shows an analysis of protein # 325 and its possible relationship to the 52 kD protein described by Müns et al. and Danilov et al.

Fig. 10 shows a strategy as to how to go further with the analysis of the protein.

Fig. 11 shows the strategy followed in obtaining the data described in Example 1.

~~5 Fig. 12 shows the MALDI tof MS analysis of marker protein 27.~~

Fig. 13 shows the MALDI tof MS analysis of marker protein 785.

#### REFERENCES

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## CLAIMS

1. A method of detecting for an individual mammal a high likelihood of having hypertension or a genetic predisposition for having hypertension, the method comprising determining  
5 the presence in a biological sample from the individual mammal of at least one marker protein which is indicative of a high likelihood of having hypertension or a genetic predisposition for having hypertension, wherein the marker protein is selected from the group consisting of

10	Marker protein	MW	pI
	27	156,000	5.85
	189	104,000	4.76
	126	93,000	5.91
	412	66,700	4.62
15	355	64,400	5.44
	352	57,500	5.73
	325	57,500	6.39
	452	53,400	5.90
	509	50,100	4.77
20	870	47,600	5.25
	1464	46,300	6.11
	603	46,000	4.79
	667	39,900	5.02
	657	38,900	5.39
25	686	37,300	4.71
	662	35,800	5.33
	711	35,100	5.49
	1180	33,600	5.28
	746	29,300	6.27
30	750	28,900	6.06
	777	27,300	4.62
	785	25,600	4.50
	803	24,600	5.28
	847	17,400	4.49
35	1031	17,300	5.70

1033	17,200	4.93
1032	16,300	5.25
862	14,900	4.65
864	14,500	4.73

- 5 and including any modifications and derivatives of these marker peptides, wherein ~~pi~~ is the isoelectric point of the marker protein as determined by isoelectric focusing, and the molecular weight (MW) is determined on a polyacrylamide gel.
2. A peptide which is not present or present in a low amount  
10 in 2D gel electrophoresis of proteins from resistance artery biopsies from hypertensive rats and which is present in a significantly higher amount in 2D gel electrophoresis of proteins from resistance artery biopsies from normotensive rats.
- 15 3. A peptide which is present in 2D gel electrophoresis of proteins from resistance artery biopsies from hypertensive rats and which is not present or present in a significantly lower amount in 2D gel electrophoresis of proteins from resistance artery biopsies from normotensive rats.
- 20 4. A peptide according to claim 3 which has a molecular weight of about 57.5 kD and an isoelectric point of about 6.39, the peptide comprising the following partial amino acid sequence Arg Ser Ala Pro Gly Leu Asn Ser Gly X X Pro Ala Glu Glu Val (SEQ ID NO:1).
- 25 5. A peptide according to claim 3 which has a molecular weight of about 57.5 kD and an isoelectric point of about 6.39, the peptide comprising the following partial amino acid sequence (Met) Leu His Glu Leu Glu Lys Ala Tyr (Arg) Phe (Lys) (SEQ ID NO:2).
- 30 6. A peptide according to claims 4 and 5 which contains both partial sequences.

7. A peptide according to claim 3 which has a molecular weight of about 17.4 kD and an isoelectric point of about 4.49, the peptide comprising the following partial amino acid sequence X Pro Thr Glu Ala Ala X (SEQ ID NO:3).
- 5 8. A peptide according to claim 3 which has a molecular weight of about 14.9 kD and an isoelectric point of about 4.65, the peptide comprising the following partial amino acid sequence Met Gln Ala Glu Met Ser Pro Ala Phe X Ser Tyr X X Gln (SEQ ID NO:4).
- 10 9. A peptide which comprises a sequence which has a degree of homology of at least 80% with an amino acid sequence selected from the group consisting of sequences SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4, irrespective of any modifications of said amino acids.
- 15 10. A peptide which comprises a sequence which differs from an amino acid sequence as defined in any of claims 2-9 in that at least one amino acid has been deleted, substituted or modified or at least one additional amino acid has been inserted so as to result in an amino acid sequence which
- 20 encodes a peptide which is a hypertension marker protein useful in the method of claim 1.
11. A peptide selected from the group consisting of
- Pro Gly Leu Asn Ser Gly X X Pro Ala Glu Glu Val (SEQ ID NO: 5)  
Pro Gly Leu Gln Ser Gly X X Pro Ala Glu Glu Val (SEQ ID NO: 6)  
25 Pro Gly Leu Asn Pro Gly X X Pro Ala Glu Glu Val (SEQ ID NO: 7)  
Pro Gly Leu Gln Pro Gly X X Pro Ala Glu Glu Val (SEQ ID NO: 8)  
Pro Gly Leu Asn Ser Gly X X Ser Ala Glu Glu Val (SEQ ID NO: 9)  
Pro Gly Leu Gln Ser Gly X X Ser Ala Glu Glu Val (SEQ ID NO: 10)  
Pro Gly Leu Asn Pro Gly X X Ser Ala Glu Glu Val (SEQ ID NO: 11)  
30 Pro Gly Leu Gln Pro Gly X X Ser Ala Glu Glu Val (SEQ ID NO: 12)  
Pro Gly Leu Asn Ser Gly X X Pro Ala Asp Glu Val (SEQ ID NO: 13)  
Pro Gly Leu Gln Ser Gly X X Pro Ala Asp Glu Val (SEQ ID NO: 14)  
Pro Gly Leu Asn Pro Gly X X Pro Ala Asp Glu Val (SEQ ID NO: 15)

- Pro Gly Leu Gln Pro Gly X X Pro Ala Asp Glu Val (SEQ ID NO: 16)  
 Pro Gly Leu Asn Ser Gly X X Ser Ala Asp Glu Val (SEQ ID NO: 17)  
 Pro Gly Leu Gln Ser Gly X X Ser Ala Asp Glu Val (SEQ ID NO: 18)  
 Pro Gly Leu Asn Pro Gly X X Ser Ala Asp Glu Val (SEQ ID NO: 19)  
 5 Pro Gly Leu Gln Pro Gly X X Ser Ala Asp Glu Val (SEQ ID NO: 20)  
 Pro Gly Leu Asn Ser Gly X X Pro Ala Glu Glu Ala (SEQ ID NO: 21)  
~~Pro Gly Leu Gln Ser Gly X X Pro Ala Glu Glu Ala (SEQ ID NO: 22)~~  
 Pro Gly Leu Asn Pro Gly X X Pro Ala Glu Glu Ala (SEQ ID NO: 23)  
 Pro Gly Leu Gln Pro Gly X X Pro Ala Glu Glu Ala (SEQ ID NO: 24)  
 10 Pro Gly Leu Asn Ser Gly X X Ser Ala Glu Glu Ala (SEQ ID NO: 25)  
 Pro Gly Leu Gln Ser Gly X X Ser Ala Glu Glu Ala (SEQ ID NO: 26)  
 Pro Gly Leu Asn Pro Gly X X Ser Ala Glu Glu Ala (SEQ ID NO: 27)  
 Pro Gly Leu Gln Pro Gly X X Ser Ala Glu Glu Ala (SEQ ID NO: 28)  
 Pro Gly Leu Asn Ser Gly X X Pro Ala Asp Glu Ala (SEQ ID NO: 29)  
 15 Pro Gly Leu Asn Pro Gly X X Pro Ala Asp Glu Ala (SEQ ID NO: 30)  
 Pro Gly Leu Gln Pro Gly X X Pro Ala Asp Glu Ala (SEQ ID NO: 31)  
 Pro Gly Leu Gln Ser Gly X X Pro Ala Asp Glu Ala (SEQ ID NO: 32)  
 Pro Gly Leu Asn Ser Gly X X Ser Ala Asp Glu Ala (SEQ ID NO: 33)  
 Pro Gly Leu Gln Ser Gly X X Ser Ala Asp Glu Ala (SEQ ID NO: 34)  
 20 Pro Gly Leu Asn Pro Gly X X Ser Ala Asp Glu Ala (SEQ ID NO: 35)  
 Pro Gly Leu Gln Pro Gly X X Ser Ala Asp Glu Ala (SEQ ID NO: 36)

12. A nucleic acid fragment comprising a nucleotide sequence which codes for a peptide shown in Table 1 or a peptide according to any of claims 2-11.

- 25 13. A nucleic acid fragment which hybridizes with a nucleic acid fragment according to claim 12 or a part thereof under stringent hybridization conditions.

14. Use of a nucleic acid fragment according to claim 12 or 13 for detecting the presence of a peptide shown in Table 1  
 30 or a peptide according to any of claims 2-11.

15. A binding means which specifically binds to a peptide shown in Table 1 or a peptide according to any of claims 2-11 or a nucleic acid fragment according to any of claims 12 or 13.

16. A binding means according to claim 15 which is an antibody or a binding fragment thereof.

17. An antibody according to claim 16 which is a polyclonal antibody.

5 18. An antibody according to claim 16 which is a monoclonal antibody.

19. Use of a binding means according to claim 15 or 16 or for detecting the presence of a peptide shown in Table 1 or a peptide according to any of claims 2-11.

10 20. A test kit for detecting whether an individual mammal has a high likelihood of having hypertension or a genetic predisposition for having hypertension, comprising:

a) binding means which specifically binds to at least one marker protein shown in Table 1 or at least one peptide  
15 according to any of claims 2-11 or a nucleic acid fragment according to any of claims 12 or 13,

b) means for detecting binding, if any, or the level of binding, of the binding means to at least one of the marker proteins or at least one of the peptides or at least one of  
20 the nucleic acid fragments, and

c) means for correlating whether binding, if any, or the level of binding, to said binding means is indicative of the individual mammal having a significantly higher likelihood of having hypertension or a genetic predisposition for having  
25 hypertension.

21. A test kit for detecting whether an individual mammal has a high likelihood of having hypertension or a genetic predisposition for having hypertension, comprising:

a) a nucleic acid according to claim 12 or 13,

b) means for detecting binding, if any, or the level of binding of the binding means to a nucleic acid according to claim 12 or 13, and

5 c) means for correlating whether binding, if any, or the level of binding, to said nucleic acid is indicative of the individual mammal having a significantly higher likelihood of having hypertension or a genetic predisposition for having hypertension.

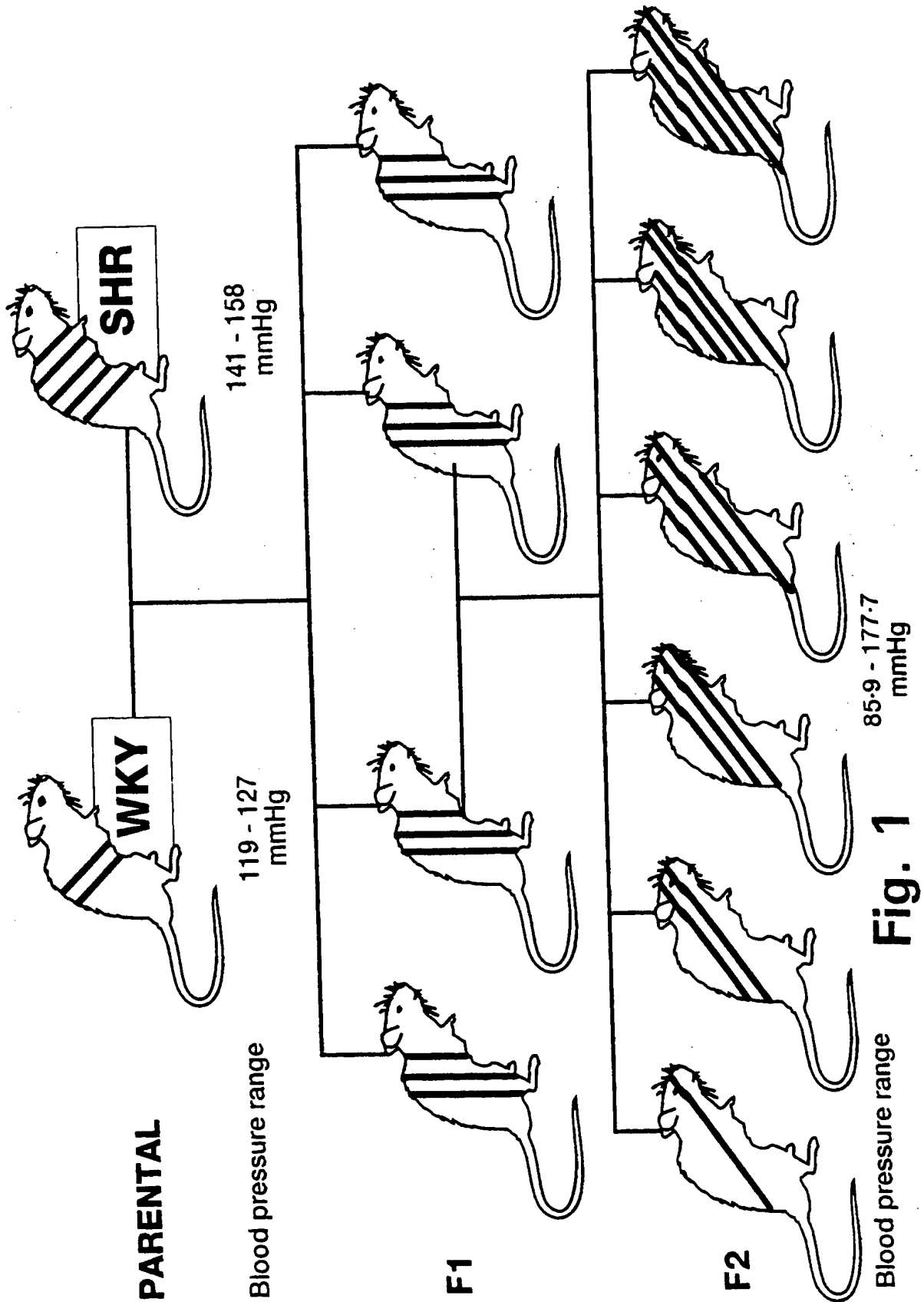
22. A method for determining the effect of a substance, the  
10 method comprising using a mammal which has been established to be an individual having a high likelihood of having hypertension or a genetic predisposition for having hypertension by use of the method of claim 1, the method comprising administering the substance to the individual and determining the  
15 effect of the substance.

23. A pharmaceutical composition which comprises a substance which is capable of regulating the expression of a nucleic acid according to claim 12 or claim 13, at least one marker protein shown in Table 1 or a peptide according to any of  
20 claims 2-11 or the activity of at least one marker protein shown in Table 1 or a peptide according to any of claims 2-11 and/or a nucleic acid according to claim 12 or claim 13.

24. A pharmaceutical composition comprising at least one marker protein shown in Table 1 or a peptide according to any  
25 of claims 2-11, a nucleic acid fragment according to claim 12 or 13 or a binding means according to claim 15.

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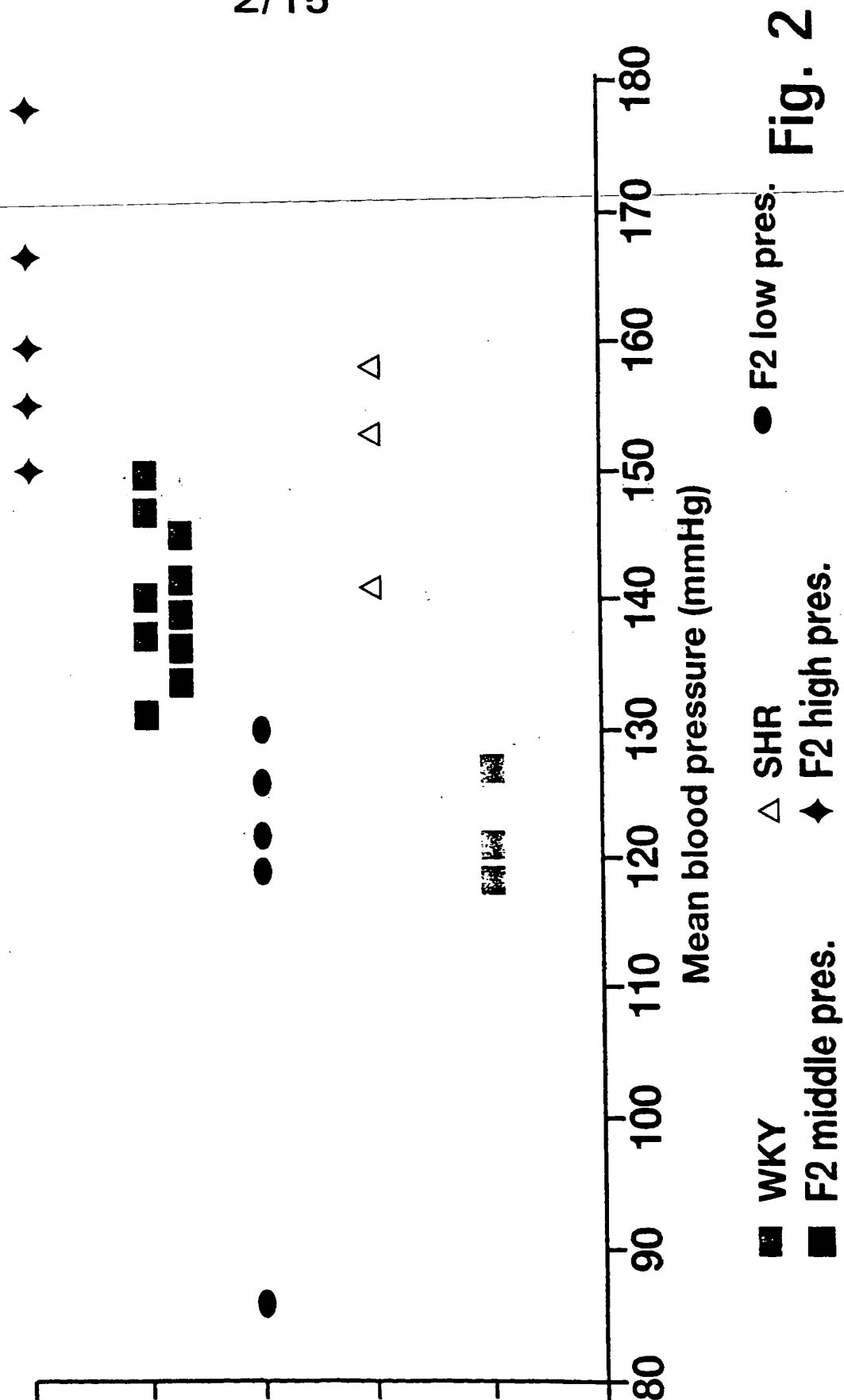
# GENETIC RELATIONSHIPS



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# EXPERIMENTAL SETUP

## BLOOD PRESSURE DISTRIBUTION





# Fig. 3

## RAW DATA

### Protein Expression in Normal & Hypertensive Rats

Prot #	Normotensive Rats (Gp1)						Hypertensive Rats (Gp3)						t value						
	9	25	34	50	55	AVG		STDS		2	3	7		11	41	AVG		STDS	
						Gp1	Gp1	Gp1	Gp1							Gp3	Gp3	Gp3	Gp3
1	0.231	0.232	0.302	0.304	0.202	0.254	0.046	0.332	0.374	0.387	0.499	0.165	0.351	0.121	1.68				
2	0.167	0.159	0.074	0.126	0.171	0.139	0.041	0.161	0.081	0.097	0.067	0.050	0.091	0.043	1.83				
3	0.033	0.021	0.024	0.018	0.039	0.027	0.009	0.070	0.047	0.022	0.028	0.015	0.036	0.022	0.88				
4	0.167	0.092	0.139	0.103	0.312	0.163	0.089	0.226	0.249	0.062	0.095	0.143	0.155	0.081	0.14				
5	0.387	0.328	0.322	0.358	0.421	0.363	0.041	0.371	0.382	0.288	0.358	0.238	0.327	0.062	1.07				
6	0.065	0.054	0.069	0.061	0.073	0.064	0.007	0.060	0.086	0.034	0.065	0.043	0.058	0.020	0.71				
7	0.05	0.094	0.206	0.104	0.123	0.115	0.057	0.137	0.202	0.095	0.107	0.065	0.121	0.052	0.17				
8	0.032	0.119	0.219	0.141	0.343	0.171	0.117	0.085	0.180	0.726	0.073	0.258	0.264	0.269	0.71				
9	0.052	0.074	0.025	0.059	0.135	0.069	0.041	0.053	0.110	0.104	0.082	0.064	0.083	0.025	0.64				
10	0.072	0.019	0.023	0.02	0.026	0.032	0.023	0.074	0.108	0.024	0.030	0.034	0.054	0.036	1.16				
11	0.491	0.407	0.355	0.538	0.61	0.480	0.102	0.553	0.840	0.459	0.433	0.725	0.602	0.175	1.34				
12	0.322	0.726	0.588	0.439	0.646	0.544	0.163	0.414	0.414	0.513	0.239	0.342	0.384	0.102	1.86				
13	0.616	0.302	0.451	0.501	0.63	0.500	0.134	0.605	0.609	0.664	0.535	0.339	0.550	0.127	0.61				
14	0.06	0.028	0.05	0.037	0.061	0.047	0.014	0.065	0.047	0.054	0.027	0.020	0.043	0.019	0.43				
15	0.291	0.815	0.388	0.542	0.573	0.522	0.200	0.285	0.314	0.788	0.400	0.397	0.437	0.203	0.67				
16	0.082	0.051	0.064	0.058	0.035	0.058	0.017	0.059	0.063	0.044	0.081	0.025	0.054	0.021	0.30				
17	1.591	0.513	0.509	0.966	0.477	0.811	0.481	0.476	0.862	0.715	1.748	0.176	0.795	0.592	0.05				
18	0.528	0.348	0.323	0.35	0.428	0.395	0.084	0.296	0.399	0.217	0.518	0.091	0.304	0.164	1.11				
19	0.856	1.064	1.501	2.007	2.505	1.587	0.677	1.632	1.979	0.866	1.619	2.132	1.646	0.489	0.16				
20	0.105	0.029	0.175	0.108	0.139	0.111	0.054	0.065	0.159	0.063	0.054	0.073	0.083	0.043	0.92				
1386	0.062	0.074	0.118	0.065	0.007	0.065	0.040	0.007	0.073	0.131	0.055	0.081	0.069	0.045	0.16				
1387	0.025	0.019	0.031	0.036	0.033	0.029	0.007	0.055	0.037	0.010	0.027	0.009	0.028	0.019	0.13				
1388	0.029	0.028	0.012	0.04	0.02	0.026	0.010	0.080	0.035	0.031	0.019	0.015	0.036	0.026	0.81				

# TEST THE HYPOTHESIS

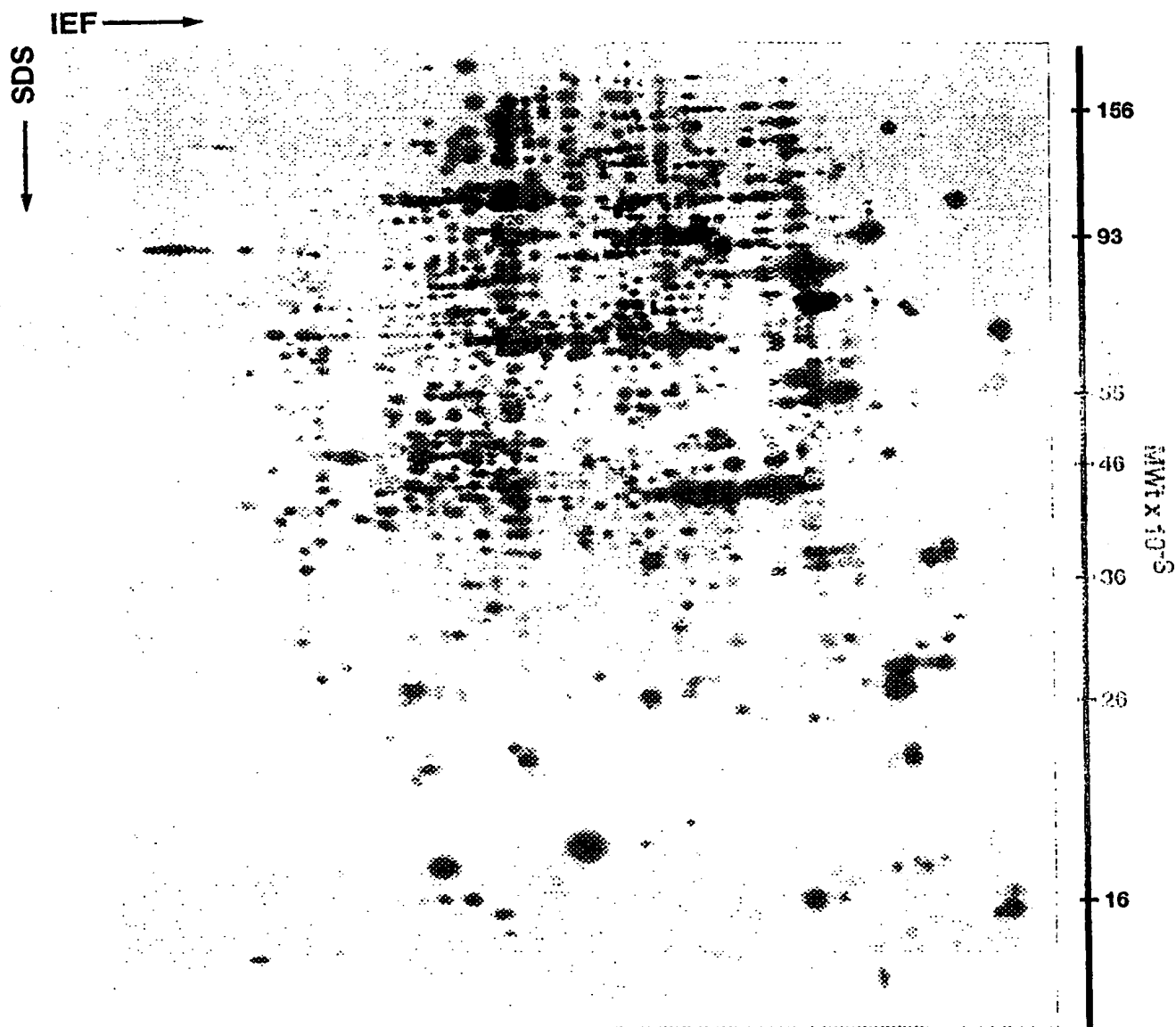
F2 generation					Parental generation					
normotensive		hypertensive		t-value	WKY		SHR		P	
%-IOD	Std	%-IOD	Std		%-IOD	Std	%-IOD	Std		
0.018	0.005	0.005	0.003	4.87	0.018	0.006	0.007	0.001	3.24	0.018
0.031	0.006	0.015	0.006	4.26	0.019	0.007	0.017	0.005	0.43	NS
0.008	0.002	0.003	0.002	4.06	0.003	0.001	0.002	0.001	2.67	0.037
0.009	0.003	0.002	0.001	3.97	0.006	0.006	0.009	0.003	0.89	NS
0.014	0.007	0.030	0.006	3.94	0.017	0.011	0.022	0.007	0.70	NS
0.001	0.001	0.004	0.002	3.61	0.002	0.001	0.002	0.001	1.29	NS
0.046	0.008	0.125	0.054	3.20	0.034	0.024	0.029	0.013	0.31	NS
0.064	0.017	0.037	0.007	3.17	0.044	0.012	0.025	0.005	2.55	0.043
0.027	0.004	0.016	0.017	3.13	0.028	0.008	0.012	0.010	2.51	0.046
0.001	0.001	0.006	0.004	3.11	0.006	0.002	0.003	0.002	1.84	NS

**Fig. 4**

**SUBSTITUTE SHEET (RULE 26)**

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# HYPERTENSION MARKERS



○ Down regulated from normotensive to hypertensive.

● Up regulated from normotensive to hypertensive.

Numbers are internal computer reference numbers.

Fig. 5

PROTEIN 847

IEF →

SDS ↓

PARENTAL  
t = 3 24

F2  
t = 4 87

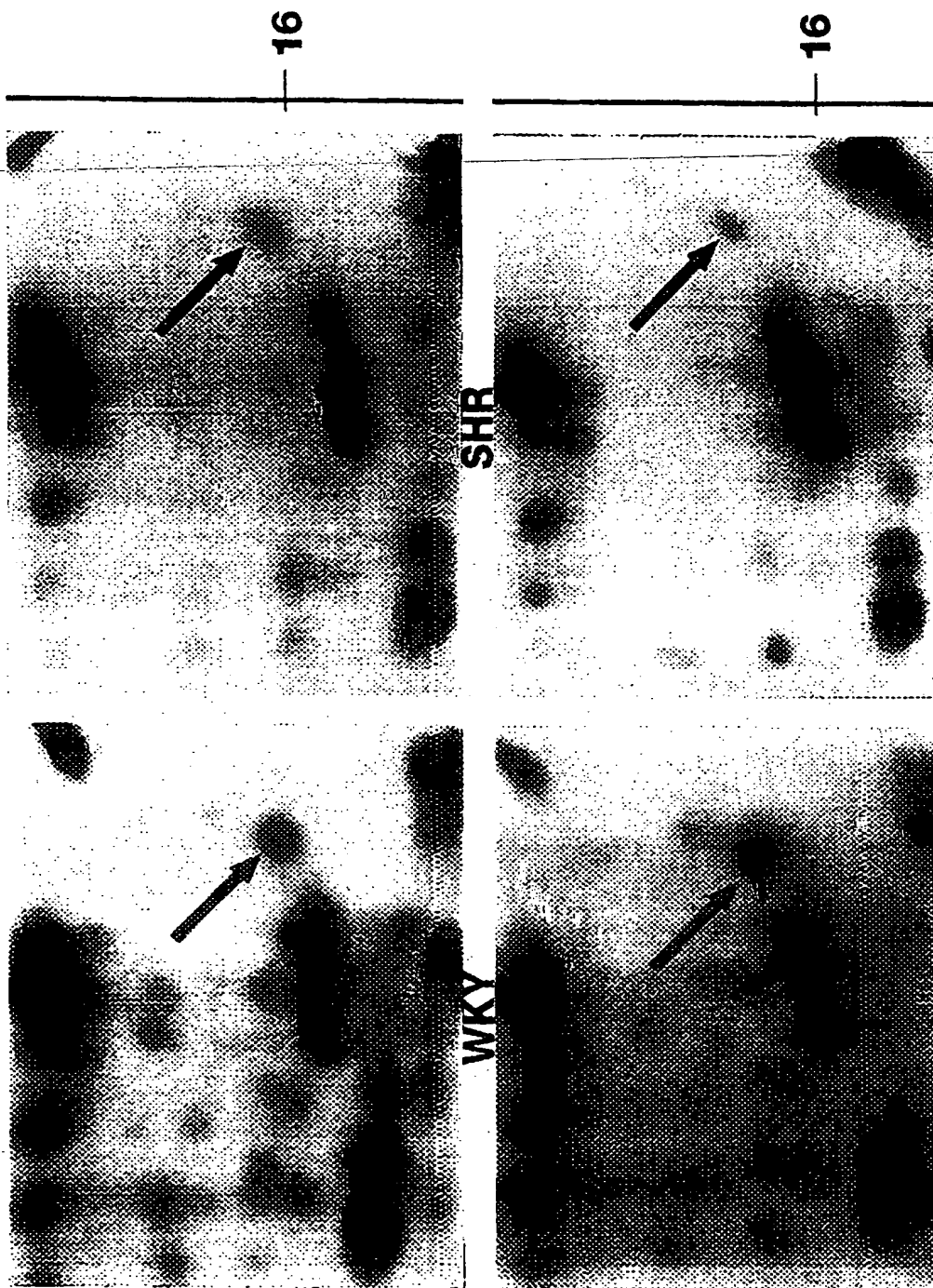


Fig. 6

# Protein #325 compared to ACE

## #325 Sequence:

Rat Wistar-Kyoto

R S A P G L I I S G X X P A I E V

## ACE:

Rat Wistar-Kyoto

A P A L D P G L Q P G N F S A D E A G A Q I L F A

Rat Norvegicus

A P A L D P G L Q P G N F S A D E A G A Q I L F A

Mouse

A P A L D P G L Q P G N F S P D E A G A Q L F A

Rabbit

A L I L D P G L L P G I F A A D E A G A R I L F A

Human

A L A L D P G L Q P G N F S A D E A G A Q L F A

Bovine

E L D P A L Q P G N F P A D E A G A Q I F A

Fig. 7

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# ANGIOTENSIN CONVERTING ENZYME (ACE)

## Functions

Angiotensinogen

Protein

↓ Renin

DRVYIHPFHL

Angiotensin I  
in-active

↓ ACE

DRVYIHPF

Angiotensin II  
active  
vasopressor

Protein

Bradykinin

RPFGSPFR

active  
vasodilator

↓ ACE

in-activated by  
proteolysis

Fig. 8

# Fig. 9

## PROTEIN #325 COMPARED TO ACE

Western blotting <sup>1</sup> Injection into SHR rats <sup>2</sup>	
Monoclonal Ab(ACE)	175kD
Polyclonal Ab(ACE)	175, 52(strong) & 47kD
	No effect
	Lethal

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- 1): G. Müns et al.; J. Cel. Biochem.: 53:352-359, 1993  
 2): S.M. Danilov et al.; Lab. Invest.: 64:118-124, 1991

### Hypothesis:

- Protein #325**
- has one similar active domain
  - is identical to the 52kD protein
  - replaces ACE's function in SHR rats

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# HOW TO GO FURTHER

Sequence from protein #325: R S A P G L N S G X X P A E E V

Is protein #325 the same as the 52kD protein?

Analyse the polyclonal ACE antibody from Dr. Müns (2DGE & western blot)

⇒ YES: analyse human lung tissue and resistance arteries (2DGE & western blot)

Find the gene for protein #325/52kD(?)

Synthesising degenerate oligonucleotide probes

⇒ search in cDNA library → sequence gene

⇒ search in human cDNA library → sequence gene

⇒ express protein #325 (in yeast) → make poly- and MoAb

⇒ function studies on human resistance arteries in myograph using antibodies

⇒ construct inducible transgenic animal to test gene function

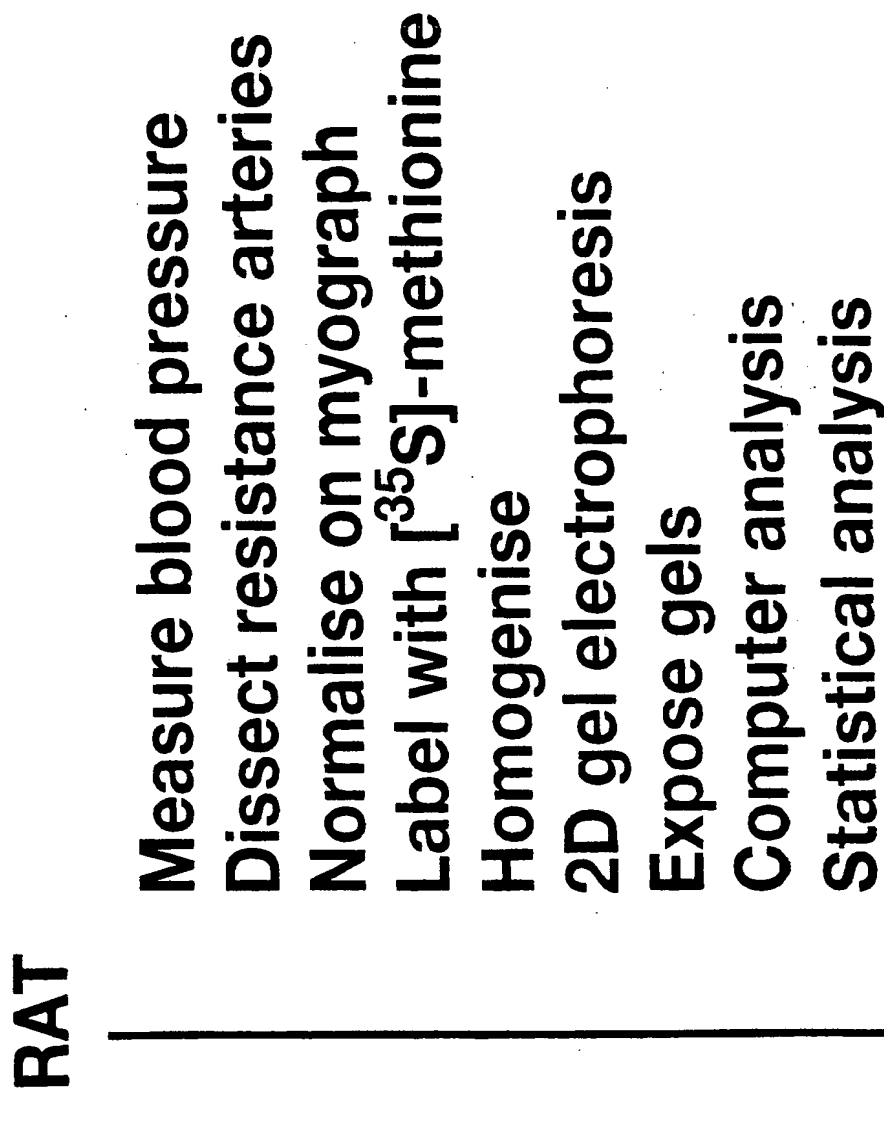
**For the other hypertension markers:**

Obtain more material for microsequencing!



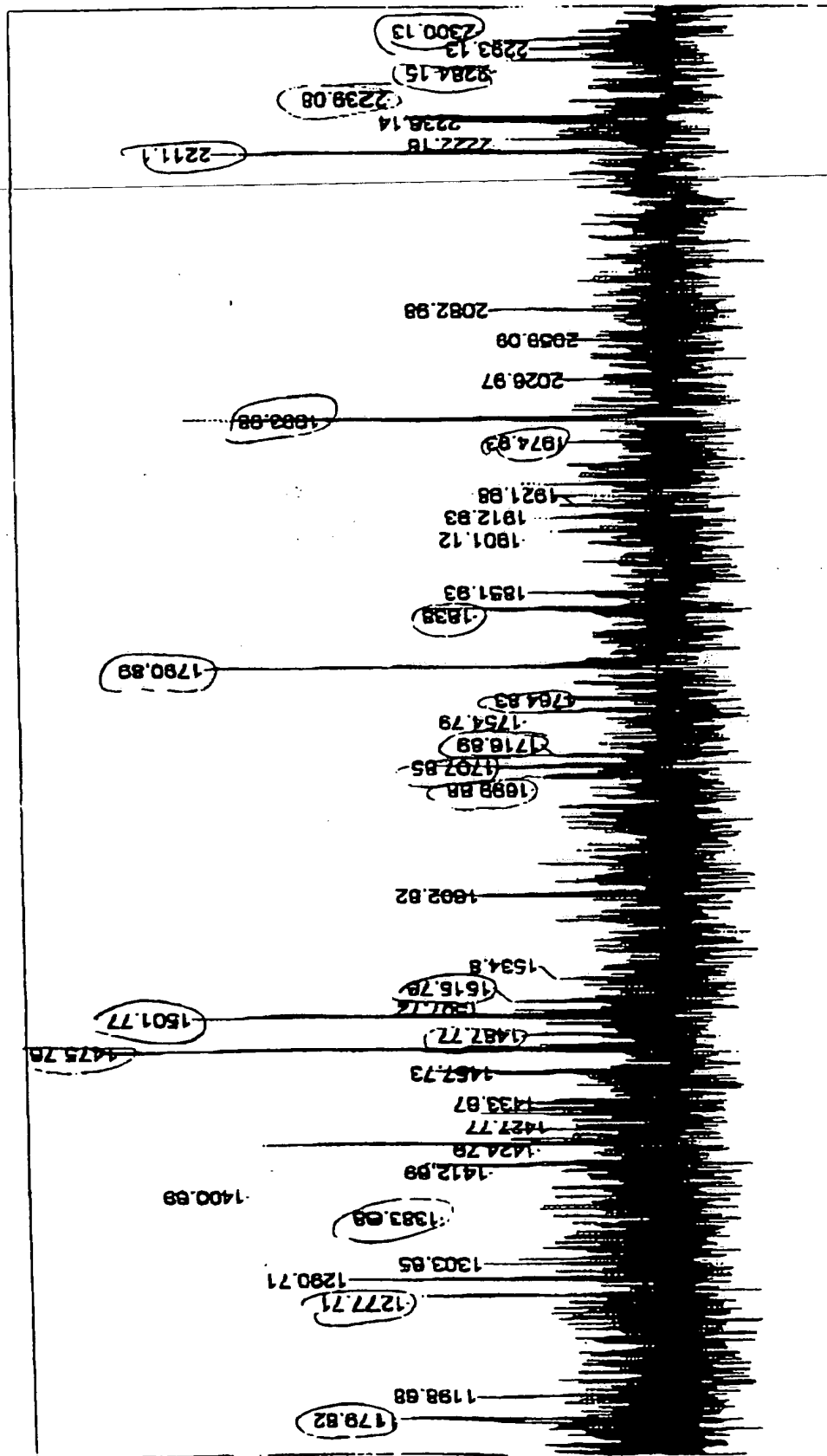
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# FROM RAT TO RESULT



**RESULT**      Fig. 11

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Mirror Ratio: 1.040  
PSD Mirror Ratio:  
Timed Ion Selector: 2000.0 OFF  
Negative Ions: OFF

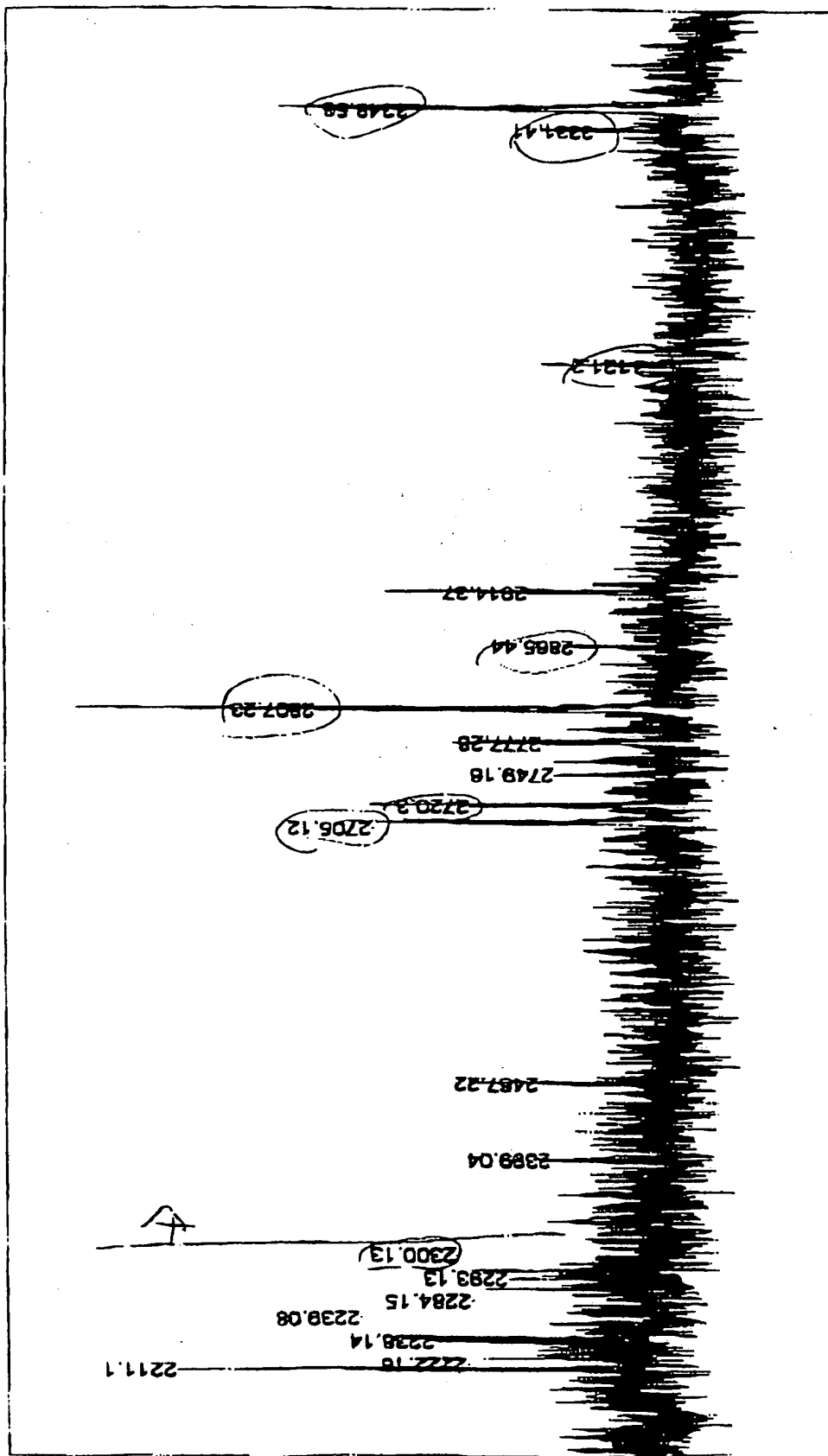
Laser : 1830  
Scans Averaged: 256  
Pressure: 5.10e-08  
Low Mass Gate: 800.0

Accelerating Voltage: 20000  
Grid Voltage: 71.500 %  
Guide Wire Voltage: 0.050 %  
Delay: 250 ON

Comment: Rat 27 micro 20 %  
Method: REFDE208  
Mode: Reflector

Fig. 12a

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Comment: Ref 27 micro 20 %

Method: REFDE20B

Mode: Reflector

Accelerating Voltage: 20000

Grid Voltage: 71.500 %

Guide Wire Voltage: 0.050 %

Delay: 250 ON

Laser: 1830

Scans Averaged: 256

Pressure: 5.10e-08

Low Mass Gate: 800.0

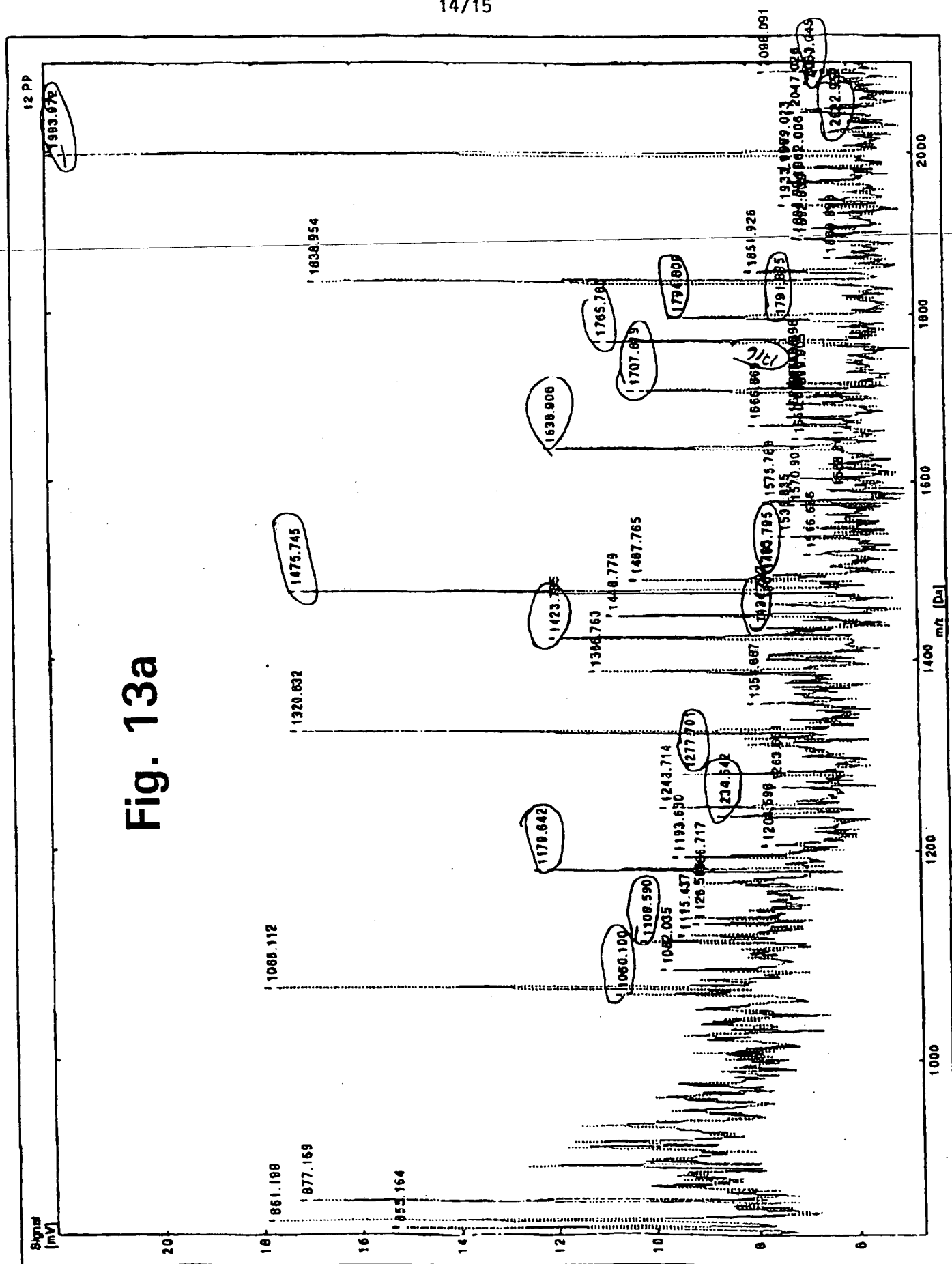
Mirror Ratio: 1.040

PSD Mirror Ratio:

Timed Ion Selector: 2000.0 OFF

Negative Ions: OFF

Fig. 12b



SUBSTITUTE SHEET (RULE 26)



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 97/00429

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 C12N9/48 C07K14/47 G01N33/68 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 09232 A (VAN ALSTYNE ET AL.) 6 April 1995 see especially claim 34, sequence xiii	7,12-14, 18
X	P A O WEERINK ET AL.: "Production of an specific antibody against pyruvate kinase type M2 using a synthetic peptide " FEBS LETTERS, vol. 236, no. 2, 29 August 1988, AMSTERDAM NL, pages 391-395, XP002037586 see figure 1 --- -/--	7,12-14, 18



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

10 March 1998

Date of mailing of the international search report

17. 03. 98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Masturzo, P

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 97/00429

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	File Medline, abstract 96054310, 1996 XP002037587 & S HUDECOVA ET AL.: "DNA analysis in the determination of a predisposition for hypertension" BRATISLAVSKE LEKARSKE LISTY, vol. 96, no. 6, June 1995, pages 322-325, see abstract ---	1-3
X	BIOLOGICAL ABSTRACTS, vol. 95, 1995 Philadelphia, PA, US; abstract no. 345279, S B HARRAP ET AL.: "The S-A gene; predisposition to hypertension and renal function in man" XP002037588 & CLINICAL SCIENCE, vol. 88, no. 6, 1995, LONDON, pages 665-670, see abstract ---	1-3
X	BIOLOGICAL ABSTRACTS, vol. 92, 1992 Philadelphia, PA, US; abstract no. 347420, XP002037589 & G C M WATT ET AL.: "Abnormalities of glucocorticoid metabolism and the renin angiotensin system; a four corners approach to the identification of genetic determinants of blood pressure" J. HYPERTENS., vol. 10, no. 5, 1992, pages 473-482, see abstract ---	1-3
A	WO 90 03435 A (INSERM) 5 April 1990 see the whole document -----	1-24

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 1-3,10,12-24

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The applicant has formulated claims 1-3 in a very vague and imprecise way. He has chosen to define the scope of these claims mostly by features which cannot be investigated by the Search Division. This makes a complete search impossible for economical reasons.

Therefore the search was limited to the claims containing a (partial) structure and to the dependent claims only insofar as they refer to these structures.

Remark : Although claim 22 is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK 97/00429

## **Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## **Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 97/00429

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		DE 68922297 T	14-09-95
		EP 0388466 A	26-09-90
		JP 3502643 T	20-06-91
		US 5480793 A	02-01-96
US 5359045 A	25-10-94		
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